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Factor VIII and IX Gene Polymorphisms and Carrier Analysis in Indian Population

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The efficacy of the three common intra- and extragenic polymorphic sites of the factor VIII and IX genes has been examined in the Indian population, with an aim to develop a strategy that would be accurate and informative, yet economical. The approach for hemophilia A carrier detection includes tests for *Bcl*I, *Xba*I, and *Taq*I polymorphic sites for introns 18 and 22 and the extragenic locus St 14, respectively, whereas for hemophilia B, tests include detection of *Taq*I, *Dde*I, and *Hha*I polymorphic sites for introns 4 and 1, and the 3′ flanking region of the factor IX gene, respectively. In hemophilia A, the cumulative efficiency of these three polymorphisms has been found to be 100%, since all 37 tested families were informative for at least one of these three polymorphisms. It is of interest to note that a case of recombination between St 14 and the factor VIII gene was also observed. Of the 47 unrelated X chromosomes examined (normal = 10, factor VIII:C deficiency = 37), heterozygosity for *Bcl*I, *Xba*I, and St 14 was found to be 47%, 36%, and 86%, respectively, in the factor VIII gene. However, when 37 unrelated X chromosomes (normal = 10, factor IX:C = 27) were analyzed for polymorphism with *Taq*I, *Dde*I, and *Hha*I, it was found that the polymorphism detection rate was only 18% for the *Taq*I site but 45% each for the *Dde*I and *Hha*I sites, in the factor IX gene. This indicates a low effectiveness of the *Taq*I restriction site in carrier analysis of hemophilia B families in our population.
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Key words: hemophilia; polymorphism; carrier detection

INTRODUCTION

In a developing country like India, carrier detection and prenatal diagnosis of hemophilia will go a long way in reducing the burden of hemophilia care in the society.

Direct detection of mutations would obviously be the method of choice for carrier detection and prenatal diagnosis of hemophilia A and B. However, due to the high heterogeneity of mutations in hemophilia, and the size and structural complexity of factor VIII and IX genes, direct diagnosis of the molecular defect causing hemophilia is often difficult, and linkage analysis with restriction fragment-length polymorphism (RFLP) is the most useful approach.

There are two possible limitations of linkage analysis in prenatal diagnosis: 1) recombination between the mutant gene and the polymorphic marker, and 2) the rate of informativeness of the marker, i.e., homozygosity of the women at the marker site, resulting in failure to discriminate the mutant gene from the normal gene. The risk of recombination is directly related to the distance between the marker and the mutation, while informativeness depends on the prevalence of the polymorphic site in a given population. Despite these potential limitations, the combined use of all available intra- and extragenic polymorphic sites makes an early diagnosis feasible in the great majority of families at risk, even though confirmation on fetal blood is sometimes necessary. Furthermore, there is no information available regarding either the pattern of mutation in hemophilia A and B or the heterozygosity rate in the Indian population.

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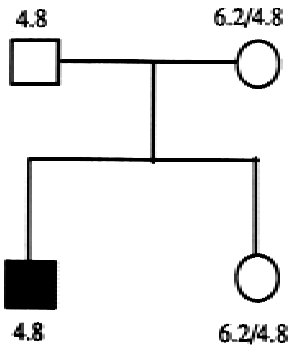
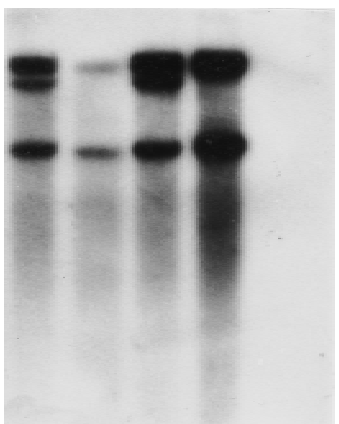
A

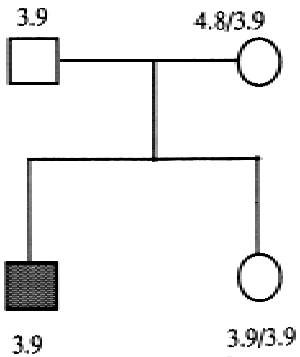
Carrier Detection by XbaI/KpnI Polymorphism

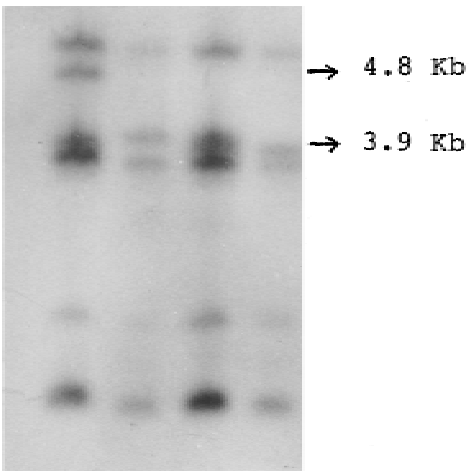




B

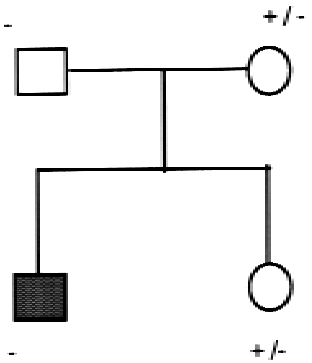
Carrier Detection by TaqI/StI4 Polymorphism





C

Carrier Detection by BclI Polymorphism



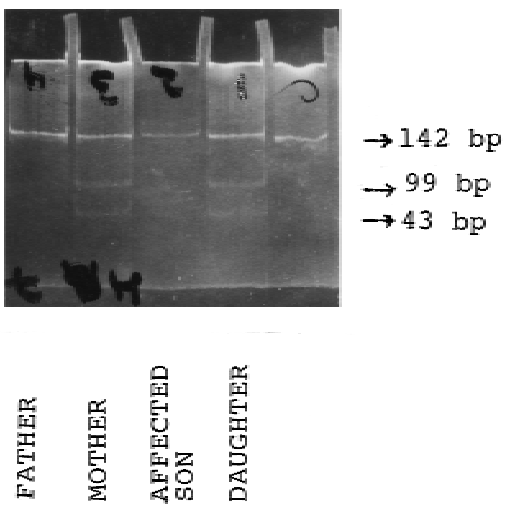


TABLE I. Restriction Enzyme Analysis of Factor VIII and Factor IX Genes

Gene	Site	Restriction enzyme	Allele	Frequency	Expected hetero-zygosity	Observed hetero-zygosity	Number of families analyzed
Factor VIII	Intron 18	*Bcl*I	142 bp	0.59	0.48	0.47	21
			99 + 43 bp	0.41			
	Intron 22	*Xba*I/*Kpn*I	6.2 kb	0.29	0.41	0.36	13
			4.8 kb	0.71			
	St 14	*Taq*I	6.6 kb	0			
			5.3 kb	0.05			
			4.8 kb	0.05			
			4.5 kb	0.35	0.87	0.86	16
			4.1 kb	0.23			
			4.0 kb				
			3.9 kb	0.21			
			3.4 kb	0.07			
Factor IX	Intron 4	*Taq*I	1.8 kb	0.87			
			1.3 kb	0.13	0.23	0.18	6
	Intron 1	*Dde*I	319 bp	0.56			
			369 bp	0.44	0.49	0.45	8
	3′ flanking region	*Hha*I	230 bp	0.62			
			150 + 80 bp	0.38	0.50	0.45	10

Since the heterozygosity rates for different RFLPs differ in different ethnic groups, we examined the utility rates of the three common polymorphic sites each of the factor VIII and IX genes i.e., the *Bcl*I, *Xba*I, and extragenic *Taq*I sites of the factor VIII gene, and the *Taq*I, *Dde*I, and 3′ *Hha*I sites of the factor IX gene. Our results suggest a strategy for providing accurate genotype assignment for virtually all female relatives of hemophiliacs in an economic and efficient way, which is very much essential for our country.

MATERIALS AND METHODS

The study involved 37 unrelated families with hemophilia A, 27 unrelated families with hemophilia B, and 10 unrelated normal families where no family history of hemophilia or any bleeding disorders could be elicited over the last four generations.

One female from each of these families was selected for study. The females selected from the hemophilia families were obligate carriers and were mothers of the index patients. Index patients and their fathers were also studied.

Routine coagulation tests and relevant factor assays were done at least twice on each index patient to confirm the nature and severity of hemophilia. Platelet aggregation with ristocetin and Von Willebrand factor (VWF) assay on relevant patients were done to rule out Von Willebrand's disease. DNA was extracted from peripheral blood leukocytes of the individuals under study, using standard techniques [1].

*Xba*I/*Kpn*I, *Taq*I/St 14 RFLPs for the factor VIII gene and *Taq*I RFLPs for the factor IX gene were studied by Southern blotting. *Bcl*I RFLPs for the factor VIII gene, and *Dde*I and *Hha*I RFLPs for the factor IX gene were studied by polymerase chain reaction (PCR). Three families with hemophilia B were also studied for *Mnl*I and *Bam*HI RFLPs, because other RFLPs did not give adequate information to assign carrier status in these three families.

Southern Blotting

Ten μg DNA were digested with various restriction endonucleases according to the manufacturer's instructions and subjected to electrophoresis for 16 h at 25 volts in 0.8% agarose gel. DNA fragments were transferred to nylon membranes (Hybond N+, Amersham, Buckinghamshire, U.K.) by Southern blotting. Probes were generous gifts from Prof. G.G. Brownlee, Sir William Dunn School of Pathology, Oxford and Dr. J.L. Mandel, INSERM U-184, France.

Polymerase Chain Reaction

Five hundred ηg DNA were used for PCR amplification of (1) intron 18 of the factor VIII gene for the *Bcl*I

Fig. 1. Recombination in a hemophilia A family between the factor VIII gene and the St 14 locus. A: Daughter assigned a noncarrier status by *Xba*I/*Kpn*I polymorphism, showing absence of the affected 4.8-kb fragment (lane 1, at left). B: Polymorphic pattern in the same family with *Taq*I/St 14. Daughter is homozygous for the 3.9-kb allele (lane 2, from left), inherited from the mother (lane 4, from left) and father (lane 3, from left). The patient shows the 3.9-kb allele, inherited from the mother (lane 1, at left). C: Polymorphism pattern with *Bcl*I in another case, assigning a noncarrier status to the daughter. Daughter (lane 4) shows the presence of 99 and 43 bp inherited from the mother. Lane C is from a normal healthy control.

polymorphism, and (2) intron 1 and the 3′ flanking region of the factor IX gene for the *Dde*I and *Hha*I polymorphisms, respectively. The primer sequences were as previously described for various PCR amplifications [2]. The reaction was performed in a total incubation volume of 50 μl, and the PCR conditions and number of cycles were as described earlier [3–6]. The amplified samples were either run in 10% polyacrylamide gel or 2% agarose gel for resolution of amplified fragments.

RESULTS

The polymorphism heterozygosity for the different restriction sites in the factor VIII and IX genes and the number of families analyzed for carrier status by different polymorphisms are shown in Table I.

Factor VIII Gene

*Taq*I RFLPs for the factor VIII gene had eight alleles, and the frequency of heterozygosity in our population was 86%. This could have been a very useful marker for carrier detection in hemophilia A, but unfortunately, as this is an extragenic marker, occasional recombination can confound interpretation, as shown in Figure 1A, B, which shows the results from the same family with hemophilia A.

It is evident from Figure 1A that the male patient has inherited the 4.8-kb RFLP and the diseased gene from his mother. The daughter has inherited the 6.2-kb RFLP from her mother, which is not associated with the disease. Hence, as shown in Figure 1A, the daughter is not a carrier. However, the results of RFLP with *Taq*I/St 14, as shown in Figure 1B, indicate that the daughter is a carrier, since 3.9 kb from the mother, associated with the disease, are present in the daughter. This anomaly occurred because of recombination between the extragenic *Taq*I/St 14 restriction site and the healthy factor VIII gene.

*Bcl*I RFLP (intron 18) was heterozygous in 47% of our patients, followed by *Xba*I/*Kpn*I RFLP (intron 22) in 36% of our patients. Figure 1C shows a typical RFLP in hemophilia A, using *Bcl*I as the restriction enzyme. In all hemophilia A families studied, either one or the other of the three restriction enzyme-related RFLPs were heterozygous in the female, and hence informative.

Factor IX Gene

*Dde*I (intron 1) RFLP was heterozygous in 45% of the females, as also were the *Hha*I RFLPs (3′ flanking region). *Taq*I (intron 4) RFLPs yielded the lowest heterozygosity, of 18%, in our population.

DISCUSSION

India has a population of 920 million people. A conservative estimate indicates 51,000 patients with hemophilia A, and 10,000 patients with hemophilia B. Unfortunately, most of these patients have no access to high-quality hemophilia care as provided by the Western nations, mainly due to financial and logistic constraints.

Gene tracking by polymorphism analysis has limitations, particularly with regard to apparent new mutations, the possibility of mosaicism, and the question of paternity. However, the relative simplicity of the procedure and its application to all hemophiliacs, irrespective of genetic background, has made these analyses extremely useful in hemophilia genetic studies.

The intron 18 *Bcl*I restriction site in the factor VIII gene showed the maximum heterozygosity, i.e., 47% in our population, as compared to other populations reported earlier, whereas the *Xba*I/*Kpn*I restriction site showed a slightly reduced heterozygosity as compared to Caucasians, Japanese, and Chinese populations [2]. In the present series of 37 families analyzed for carrier status, all were informative, with at least one of the three polymorphisms. Carrier status in only three hemophilia A families in our series could not be confirmed by intragenic polymorphisms.

A case of recombination between the factor VIII gene and the St 14 locus was observed during the family analysis. The consultant was assigned a noncarrier status based on *Bcl*I and *Xba*I/*Kpn*I polymorphisms, but *Taq*I/St 14 analysis indicated that she was a carrier (Fig. 1). This showed the event of recombination between the extragenic locus (St 14) and factor VIII gene. Under such circumstances, the results from intragenic RFLPs may be relied upon.

The efficiency of the *Taq*I restriction site in the factor IX gene has been found to be quite low in our population, i.e., 18%, as compared to Caucasians with a heterozygosity of 45% [6]; but it is much higher than in the Japanese (0%) [7], and Chinese and Malays (0.02%) [8,9]. Of the 27 hemophilia B families analyzed with *Taq*I, *Dde*I, and *Hha*I, only three families failed to reveal any of the three polymorphic sites. However, they were assigned carrier status by *Mnl*I and *Bam*HI polymorphism segregation analysis. *Mnl*I was informative for two families, and *Bam*HI for one family.

Thus, from the present work it can be concluded that the ability of the above three polymorphic sites of the factor VIII gene to detect carrier status is almost 100% in our Indian population. Further, it can also be emphasized that one should utilize extragenic polymorphism detection when the intragenic polymorphisms are not informative. The *Taq*I site in the factor IX gene is probably the restriction site of last preference in our population, with only 18% heterozygosity. Other intragenic markers, like *Mnl*I or *Xmn*I sites, should be analyzed for heterozygosity rating and subsequently carrier analysis.

Recently, inversion analysis of intron 22 [10,11] and minisatellite DNA analysis [2] around the factor VIII

gene have been increasingly used to study hemophilia A patients and their families. Major advances have also been made in the elucidation of mutations involving factor IX gene in hemophilia B patients [12]. These techniques have yet to find their places in research as well as in clinical practice, in developing countries such as India.

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